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Cross-talk between NMDA and $GABA_A$ receptors in cultured neurons of the rat inferior colliculus

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Neuronal ion channels of different types often do not function independently but will inhibit or potentiate the activity of other types of channels, a process called cross-talk. The *N*-methyl-*D*-aspartate receptor (NMDA receptor) and the γ-aminobutyric acid type A receptor ($GABA_A$ receptor) are important excitatory and inhibitory receptors in the central nervous system, respectively. Currently, cross-talk between the NMDA receptor and the $GABA_A$ receptor, particularly in the central auditory system, is not well understood. In the present study, we investigated functional interactions between the NMDA receptor and the $GABA_A$ receptor using whole-cell patch-clamp techniques in cultured neurons from the inferior colliculus, which is an important nucleus in the central auditory system. We found that the currents induced by aspartate at 100 μmol L^{-1} were suppressed by the pre-perfusion of GABA at 100 μmol L^{-1}, indicating cross-inhibition of NMDA receptors by activation of $GABA_A$ receptors. Moreover, we found that the currents induced by GABA at 100 μmol L^{-1} (I_{GABA}) were not suppressed by the pre-perfusion of 100 μmol L^{-1} aspartate, but those induced by GABA at 3 μmol L^{-1} were suppressed, indicating concentration-dependent cross-inhibition of $GABA_A$ receptors by activation of NMDA receptors. In addition, inhibition of I_{GABA} by aspartate was not affected by blockade of voltage-dependent Ca^{2+} channels with $CdCl_2$ in a solution that contained Ca^{2+}, however, $CdCl_2$ effectively attenuated the inhibition of I_{GABA} by aspartate when it was perfused in a solution that contained Ba^{2+} instead of Ca^{2+} or a solution that contained Ca^{2+} and 10 mmol L^{-1} BAPTA, a membrane-permeable Ca^{2+} chelator, suggesting that this inhibition is mediated by Ca^{2+} influx through NMDA receptors, rather than voltage-dependent Ca^{2+} channels. Finally, KN-62, a potent inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), reduced the inhibition of I_{GABA} by aspartate, indicating the involvement of CaMKII in this cross-inhibition. Our study demonstrates a functional interaction between NMDA and $GABA_A$ receptors in the inferior colliculus of rats. The presence of cross-talk between these receptors suggests that the mechanisms underlying information processing in the central auditory system may be more complex than previously believed.

inferior colliculus, *N*-methyl-*D*-aspartate receptor, γ-aminobutyric acid type A receptor, whole-cell patch-clamp, cross-talk

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Neuronal receptor ion channels of different types are activated by specific ligands called neurotransmitters, and the activation of these channels by their corresponding neurotransmitters may appear to be an independent process. However, accumulating evidence indicates that this is not the case. The activation of specific ion channels is often

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modulated by the activation of other ion channels [1–3]. This so-called "cross-talk" between two different types of ion channels means that the activation of one channel can inhibit or potentiate the activation of another. Experimentally, cross-talk can be demonstrated by recording the channel currents from two different ion channels while specific agonists of these channels are applied simultaneously or sequentially. Previous studies have shown that a number of receptors can engage in cross-talk. For example, cross-talk occurs between adenosine triphosphate (ATP) P2X and 5-hydroxytryptamine (5-HT) receptors in rat pheochromocytoma cells [1], between glycine and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors in rat spinal neurons [2], between *N*-methyl-*D*-aspartate (NMDA) and dopamine D1 receptors [3], and between glycine and γ-aminobutyric acid (GABA) receptors in rat spinal dorsal horn neurons [4].

NMDA and $GABA_A$ receptors are the major excitatory and inhibitory synaptic receptors in the central nervous system (CNS), respectively. The NMDA receptor is one of three subtypes of ionotropic glutamate receptors, i.e., the AMPA receptor, the kainate receptor and the NMDA receptor [5,6]. The GABA type A receptor ($GABA_A$ receptor) is a principal inhibitory receptor and gate, which is distinct but homologous to a class of chloride-permeable ion channels. Activation of NMDA receptors mediates excitatory neurotransmission and plays a fundamental role in both physiological and pathological processes in the CNS. Activation of $GABA_A$ receptor leads to an inward flow of chloride ions and a hyperpolarizing neuronal response, which is crucial for normal brain function, such as nociceptive messages [7,8] and auditory signal processing [9,10]. Both NMDA and $GABA_A$ receptors are widely distributed throughout the CNS [11,12], including in the inferior colliculus (IC), which is a major synaptic integration center and relay station of the central auditory pathway in mammals. Moreover, auditory processing in the IC involves both NMDA and $GABA_A$ receptors [13,14].

It has been reported that the activation of NMDA receptors suppresses the function of $GABA_A$ receptors in rat cerebellum granule cells, in hippocampal neurons and in the rat sacral dorsal commissural nucleus. Suppression of $GABA_A$ receptor responses by the activation of NMDA receptors is a Ca^{2+}-dependent process, and enzymes such as calcineurin, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and NO-synthase are involved in the process [15–17]. Currently, however, the interaction between these two receptors has not been systematically studied. In particular, relatively little is known concerning the interaction between NMDA receptors and $GABA_A$ receptors in central auditory regions. In the present study, we investigated the cross-talk between NMDA and $GABA_A$ receptors and the possible mechanisms underlying this cross-talk in cultured IC neurons using whole-cell patch-clamp recording techniques. Our results show that activation of $GABA_A$ receptors suppresses NMDA receptor-mediated currents and activation of NMDA receptors conversely reduces $GABA_A$ receptor-mediated currents, indicating the presence of cross-talk between NMDA and $GABA_A$ receptors in the central auditory system.

1 Materials and methods

1.1 Cell culture

All procedures conducted with animals in this study followed the guidelines and protocols approved by the Institutional Animal Care and Use Committee of the University of Science and Technology of China. All efforts were made to minimize the number of animals used and any pain or distress in the animals used.

The neurons used for cell culture were dissociated from the IC of newborn Wistar rats (postnatal day 0) as previously described [18]. Briefly, the IC was dissected from the brainstem under a dissection microscope. The tissue was incubated with 0.25% trypsin (Sigma, St Louis, MO, USA) for 10 min at 37°C and mechanically dissociated by trituration with a Pasteur pipette in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA). The isolated neurons were plated (1.5×10^6 cell mL^{-1}) on poly-L-lysine (Sigma, St Louis, MO, USA)-coated cover glasses and grown for 24 h in a DMEM mixture with L-glutamine, 10% fetal bovine serum, 10% F-12 nutrient, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Gibco, USA). Then, we placed the cells in a neuron-basal medium (1.5 mL) containing 2% B27, which was replaced every 3 d. To block the division of non-neuronal cells and stabilize the cell population, we treated the neurons with 5-fluoro-5′-deoxyuridine (20 μg mL^{-1}; Sigma, USA) on the fourth day after plating. The cultures were maintained at 37°C in 5% CO_2 and 95% humidified air, and the cells were used for electrophysiological recordings 9–14 d after plating.

1.2 Electrophysiology

Whole-cell voltage-clamp recordings were carried out using a patch-clamp amplifier (Axon 200B, Axon Instruments, USA). Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PC-10, Narishige, Japan). The resistance between the recording electrode filled with pipette solution and the reference electrode was 2–5 MΩ. In the experiments, 70%–90% series resistance was compensated, and the membrane potential was held at −60 mV throughout the experiment under voltage clamp conditions. Data were sampled using a Digidata 1320A interface and analyzed using a computer installed with Clampex and Clampfit software (Version 9.0.1, Axon Instruments, USA). All experiments were performed at room temperature (22–25°C). The data were sampled at 5

kHz and low pass filtered at 2 kHz. All measurements were carried out after stabilization of the aspartate (Asp) responses or the GABA responses.

1.3 Solutions and drugs

The standard external solution consisted of (in mmol L^{-1}): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES. The pH was adjusted to 7.4 using Tris base. In some experiments, we needed to remove the $MgCl_2$ and add 1 μmol L^{-1} Gly to the external solution, replace the $CaCl_2$ with $BaCl_2$, or add 10 μmol L^{-1} of $CdCl_2$ to the external solution. The osmolarity of all bath solutions was adjusted to 310–320 mOsm L^{-1} with sucrose (model 3300; Advanced Instruments, Pomona, CA, USA).

The patch pipette solution for whole-cell patch recording consisted of (in mmol L^{-1}): 120 KCl, 30 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 5 ethylene glycol-bis-(2-aminoethylether)-tetra acetic acid (EGTA), 2 Mg-ATP, and 10 HEPES. The pH was adjusted to 7.2 with Tris base. In some experiments, 10 mmol L^{-1} BAPTA, a membrane-permeable Ca^{2+} chelator, was added to the pipette solution.

Unless otherwise specified, the drugs used in the present study were all purchased from Sigma, Inc., USA. All drugs were applied with a rapid application technique which was termed the 'Y-tube' method [19]. With this system, we can completely exchange the external solution surrounding a neuron within 20 ms.

1.4 Statistical analysis

Clampfit software (Version 9.2, Axon Instruments, USA) and Origin 8.0 software (OriginLab Corporation, USA) were used for data analysis. Statistical significance between two groups was assessed with a Student's *t*-test. $P<0.01$ was considered to be statistically significant. All the data are represented as the mean±the standard error of the mean (SEM). P and n represent the value of significance and the number of neurons, respectively.

2 Results

2.1 Activation of $GABA_A$ receptors suppressed NMDA receptor-mediated responses

Both NMDA receptor-mediated current and $GABA_A$ receptor-mediated current could be recorded in the IC neurons. In the presence of Mg^{2+}-free external solution plus 1 μmol L^{-1} glycine, Asp at 100 μmol L^{-1} induced an inward current (I_{Asp}) at the holding potential of -60 mV. This I_{Asp} could be completely blocked by 100 μmol L^{-1} APV (D-2-amino-5-phosphonovalerate), a specific NMDA receptor antagonist, indicating that the I_{Asp} recorded in our experiments is mediated by NMDA receptors. Similarly, a current evoked by application of GABA (I_{GABA}) could be completely blocked by bicuculline (10 μmol L^{-1}), a specific $GABA_A$ receptor antagonist, indicating that the I_{GABA} recorded in the IC neurons is mediated by $GABA_A$ receptors. Moreover, I_{Asp} and I_{GABA} also could be observed from the same neurons.

We observed cross-inhibition between NMDA and $GABA_A$ receptors in IC neurons. When 100 μmol L^{-1} GABA was applied following application of 100 μmol L^{-1} Asp, we found that the amplitude of I_{Asp} was 53%±3% of control 5 s after application of GABA, and the effect was reversible. Pre-perfusion of 10 μmol L^{-1} bicuculline for 3 min blocked the inhibitory effect of GABA on the I_{Asp} (Figure 1). As the time interval between sequential application

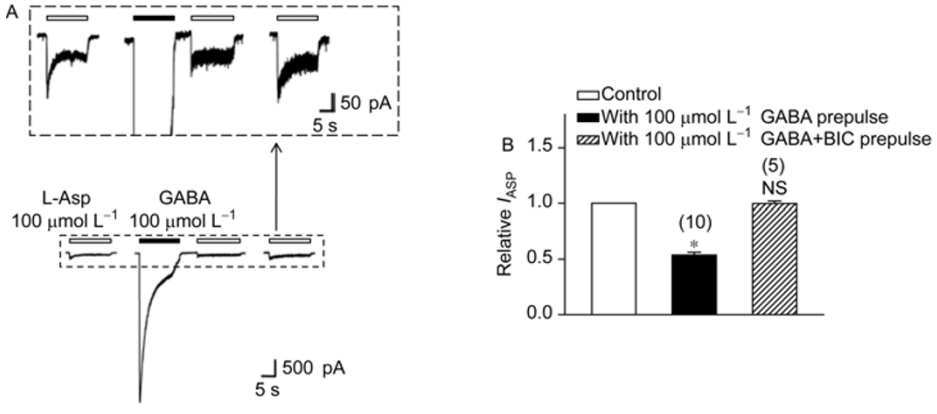

Figure 1 GABA-induced reversible suppression of I_{Asp}. A, I_{Asp} was reversibly suppressed by a preceding 100 μmol L^{-1} GABA administration in an IC neuron. The interval between GABA and Asp application was 5 s. A solid arrow indicates an enlargement of the current traces. B, Statistics showing the normalized suppression of 100 μmol L^{-1} Asp-evoked currents by preapplication of 100 μmol L^{-1} GABA or by pre-application of 100 μmol L^{-1} GABA following pre-perfusion of 10 μmol L^{-1} bicuculline for 3 min. In this and the subsequent figures, the horizontal bars indicate the time course of drug applications, vertical bars represent the mean±SEM and the number of experiments is shown in parentheses. *, $P<0.01$, NS indicates no statistically significant difference.

of GABA and Asp was increased, the inhibition progressively decreased (Figure 2), indicating that the recovery of I_{Asp} was dependent upon elapsed time.

2.2 The inhibition of I_{GABA} by activation of NMDA receptors was state-dependent

To determine whether activation of NMDA receptors also affects the activity of $GABA_A$ receptors, we examined the effects of activation of NMDA receptors on I_{GABA}. We found that activation of NMDA receptors suppressed I_{GABA}, and the inhibition of I_{GABA} was dependent on the GABA concentration. When a higher concentration of GABA (100 μmol L^{-1}) was applied, pre-application of 100 μmol L^{-1} Asp did not significantly change the amplitude of I_{GABA} over various pre-application time intervals (5 s/15 s/25 s) (Figure 3A and B). However, when a lower concentration of GABA (3 μmol L^{-1}) was applied, the amplitude of I_{GABA} was 73%±1% of control 5 s after application of 100 μmol L^{-1} Asp (Figure 3C and D). Pre-perfusion of 100 μmol L^{-1} APV for 6 min blocked I_{Asp} and the effect of Asp inhibition on I_{GABA} (Figure 3D). When we increased the time interval between the sequential application of Asp and GABA, the inhibition of I_{GABA} was no longer detectable when the interval reached 15 s (Figure 3D). Given that the recovery of I_{GABA} is rapid, we choose a time interval between the application of Asp and GABA of 5 s and a concentration of GABA of 3 μmol L^{-1} for all subsequent experiments.

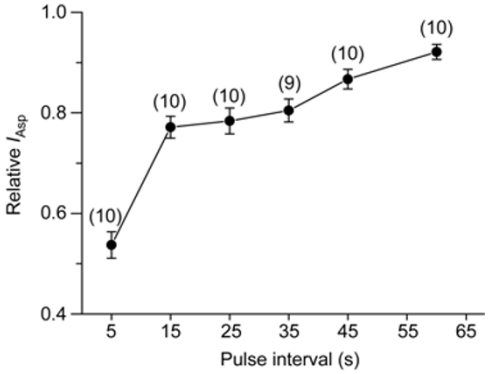

Figure 2 Time-dependent recovery of I_{Asp} expressed as the normalized amplitude of I_{Asp} and measured as the time interval between sequential application of GABA and Asp increased. All points shown are the mean±SEM.

2.3 Intracellular Ca^{2+} is involved in the suppression of the activation of NMDA receptors by I_{GABA}

Recent studies have shown that changes in the intracellular Ca^{2+} concentration influence the function of GABA receptors [20,21]. To investigate whether the Asp-induced suppression of the GABA response is Ca^{2+} dependent, we substituted the Ba^{2+} for Ca^{2+} in the external solution. In this Ca^{2+}-free extracellular solution, the Asp-induced suppression

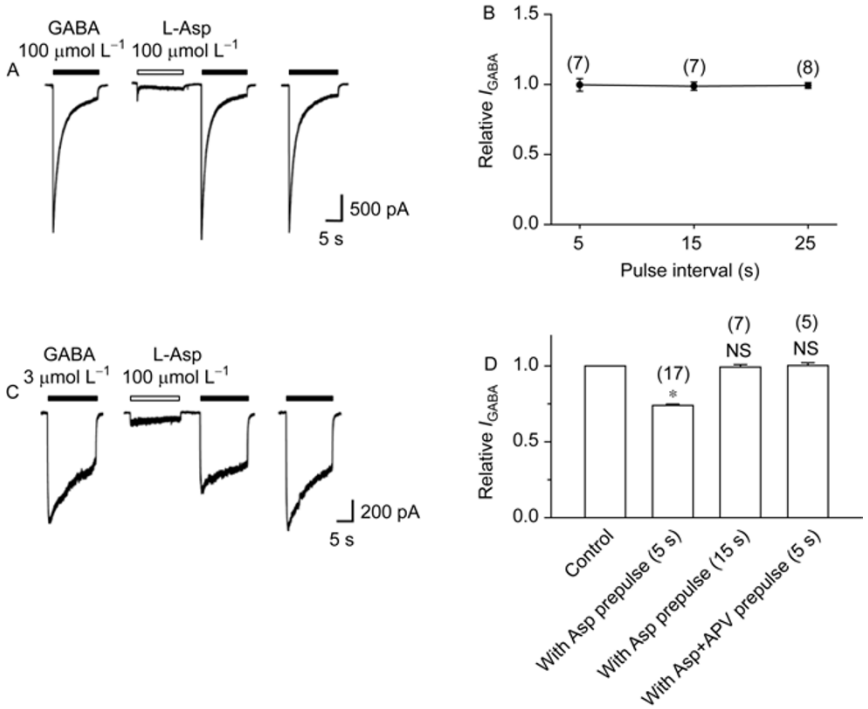

Figure 3 The effects of pre-application of Asp on I_{GABA} are state-dependent. A and B, pre-application of 100 μmol L^{-1} Asp did not significantly change the amplitude of 100 μmol L^{-1} GABA-evoked currents (I_{GABA}) over various pre-application time intervals (5 s/15 s/25 s). C and D, I_{GABA} induced by 3 μmol L^{-1} GABA were reversibly suppressed by a preceding 100 μmol L^{-1} Asp administration. The interval between Asp and GABA application was 5 s. Pre-perfusion of 100 μmol L^{-1} APV for 6 min blocked this inhibition. When the interval was increased to 15 s, the inhibition of I_{GABA} was no longer detectable. The symbols and bars in B and the vertical bars in D represent the mean±SEM. *, P<0.01, NS indicates no statistically significant difference.

of the GABA response was robustly reduced (Figure 4). Furthermore, the inhibitory effect of Asp on I_{GABA} disappeared when 10 mmol L^{-1} BAPTA was added to the pipette solution. These results indicate that an increase in the intracellular Ca^{2+} concentration contributes to the inhibition of I_{GABA}. To investigate whether Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCCs) also induces an inhibition of I_{GABA}, we added 10 μmol L^{-1} $CdCl_2$, a VDCCs blocker, to the extracellular solution. Following this manipulation, the amplitude of I_{GABA} was inhibited by the Asp pre-application to the same extent (72%±3%) as that of control (73%±1%) (Figure 4). These results suggest that the Ca^{2+} mediated inhibition is specific for Ca^{2+} influx through NMDA receptor channels, but not VDCCs.

2.4 CaMKII may mediate the suppression of I_{GABA}

Since previous studies have shown that CaMKII is involved in the suppression of I_{GABA} by activation of NMDA receptors in SDCN neurons [17], we examined the possible role CaMKII plays in the inhibition of I_{GABA} by activation of NMDA receptors in IC neurons. When stable GABA responses were obtained, and 1 μmol L^{-1} potent inhibitor of CaMKII KN-62 was perfused for 8 min, we did not observe any inhibition on I_{GABA} by application of Asp (Figure 5), indicating that CaMKII contributes to Asp-induced suppression of I_{GABA} in IC neurons.

3 Discussion

In the present study, we demonstrated that activation of the NMDA receptor and the $GABA_A$ receptor can result in a mutual inhibition between the two receptors in cultured IC neurons. These results indicate the presence of cross-talk between the NMDA and the $GABA_A$ receptors in this central

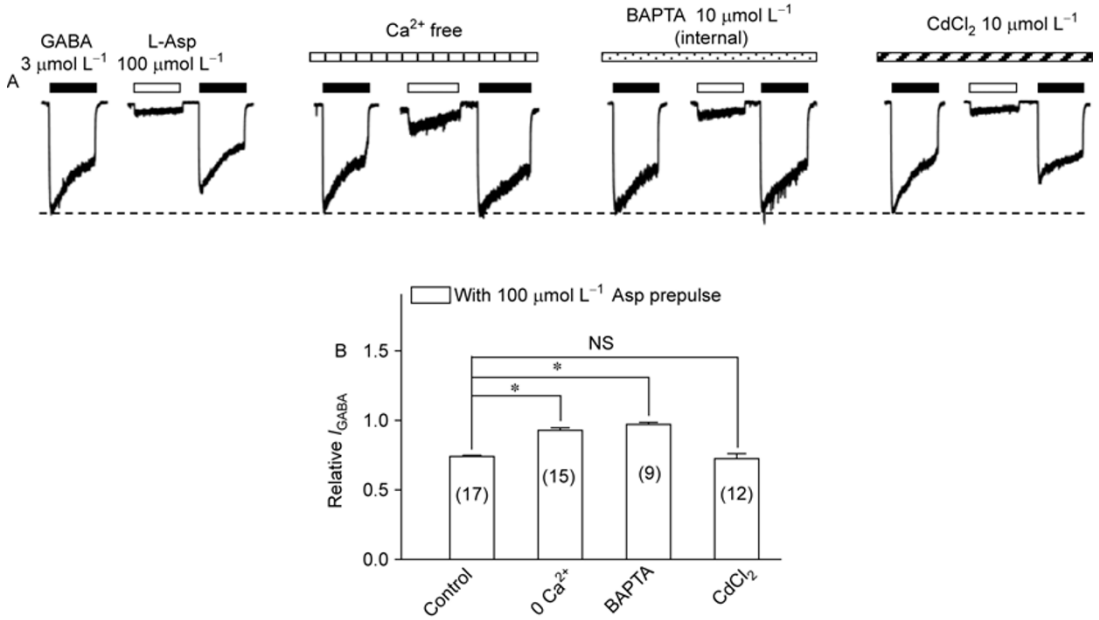

Figure 4 Involvement of intracellular Ca^{2+} in the Asp-induced depression of I_{GABA}. A, raw current traces showing a reduction in the effects of 100 μmol L^{-1} Asp on 3 μmol L^{-1} GABA in a Ca^{2+}-free bath or following addition of 10 mmol L^{-1} BAPTA. The effects of Asp were absent when 10 μmol L^{-1} $CdCl_2$ was added to the extracellular solution. The amplitudes of I_{GABA} were normalized to the amplitudes before Asp application. B, pooled percent suppression of 3 μmol L^{-1} GABA-evoked currents by 100 μmol L^{-1} Asp in the conditions indicated under the corresponding columns. Vertical bars indicate the mean±SEM. *, P<0.01, NS indicates no statistically significant difference.

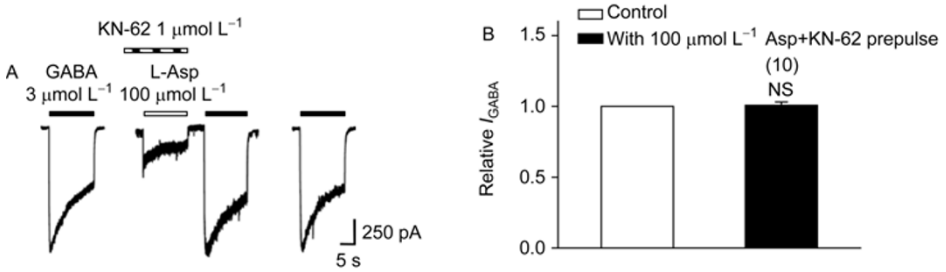

Figure 5 Involvement of CaMKII in Asp suppression of I_{GABA}. A, KN-62 (1 μmol L^{-1}), a potent inhibitor of CaMKII, markedly decreased the inhibitory effects of 100 μmol L^{-1} Asp on I_{GABA}. B, Summary of the normalized I_{GABA} in the presence of 1 μmol L^{-1} KN-62. Vertical bars represent the mean±SEM. NS indicates no statistically significant difference.

auditory region. Our findings are consistent with those reported by previous workers in other areas of the central nervous system [15,16,22]. For example, prior activation of $GABA_A$ receptors inhibits NMDA receptor-mediated whole-cell currents in cultured rat hippocampal neurons [23]. However, our study provides more detailed information regarding how the two receptors interact with each other. In this regard, our findings detail the role of VDCCs and Ca^{2+} ions in this cross-talk. Specifically, here we show that the inhibition of I_{GABA} by aspartate is not affected by blockade of VDCCs, but the inhibition is blocked when the Ba^{2+} for Ca^{2+} in the external solution or when 10 mmol L^{-1} membrane-permeable Ca^{2+} chelator BAPTA is added to the pipette solution (Figure 4), suggesting that this inhibition is mediated by Ca^{2+} influx through NMDA receptor channels rather than VDCCs. In addition, the potent inhibitor of CaMKII KN-62 reduced the inhibition of I_{GABA} by aspartate, indicating the involvement of CaMKII in the cross-inhibition. Finally, our findings suggest that the typical functions of the NMDA and GABA receptors are required for cross-talk between them because the GABA-induced suppression of I_{Asp} was attenuated by bicuculline, a specific antagonist of the $GABA_A$ receptor, and the Asp-induced suppression of I_{GABA} was attenuated by APV, a specific antagonist of the NMDA receptor.

Our results confirm the important role of Ca^{2+} ions in the modulation of NMDA receptor activity and interactions between NMDA and $GABA_A$ receptors [24,25]. It has been suggested that elevations of intracellular Ca^{2+} contribute to the inhibitory effects of NMDA receptor activation on I_{GABA} [16,17]. In neurons, the $[Ca^{2+}]_i$ is elevated by release of Ca^{2+} from intracellular stores and/or influx of extracellular Ca^{2+} through membrane ion channels. In our study, there are three observations that indicate the involvement of an influx of Ca^{2+} through NMDA receptors in the inhibition of I_{GABA}. First, in the absence of extracellular Ca^{2+}, the Asp-mediated inhibition of I_{GABA} diminished. Second, strong buffering of $[Ca^{2+}]_i$ through addition of BAPTA prevented Asp-induced suppression of I_{GABA}. Third, consistent with previous findings, Asp-induced suppression of I_{GABA} did not require the activation of VDCCs because Asp still inhibited I_{GABA} even after Cd^{2+} blocked the VDCCs [20,24,26]. It is probable that $[Ca^{2+}]_i$ entering through VDCCs cannot rise to a sufficient level to affect I_{GABA}.

What are the downstream signaling events involved in the cross-inhibition between $GABA_A$ and NMDA receptors? In hippocampal cells, a study has shown that intracellular Ca^{2+} suppresses the GABA-evoked response by activating a phosphatase [27]. However, other studies have shown that dephosphorylation is involved in NMDA-induced suppression of the GABA-evoked response [15,16]. In our studies, pretreatment with KN-62, a potent inhibitor of CaMKII, completely abolished the inhibition of Asp on I_{GABA} (Figure 5), indicating that the elevation of $[Ca^{2+}]_i$ induced by NMDA receptor activation could suppress the GABA response by activation of CaMKII, which is a multifunctional enzyme which could catalyze the phosphorylation of various proteins, such as nitric oxide synthase, GABA-modulin, $GABA_A$ receptors, phospholipase A2, acetyl-CoA carboxylase and calcineurin [28]. Based on our findings, we believe that the activation of CaMKII by the elevation of $[Ca^{2+}]_i$ may suppress the GABA response through phosphorylation of sites on $GABA_A$ receptors, such as on the beta 1 subunit or the gamma 2L/2S subunits [29]. In our experiments, the suppression of I_{GABA} by Asp gradually diminished 15 s after the removal of Asp. This transient effect of Asp inhibition on I_{GABA} may be because of transient phosphorylation by activated CaMKII. Meanwhile, Ca^{2+} and calmodulin may activate intracellular Ca^{2+}-dependent protein phosphatases and allow for a rapid recovery of I_{GABA} through dephosphorylation [30].

The mechanism underlying the GABA-induced suppression of I_{Asp} has not been fully established. It has been shown the direct protein-protein coupling enables functional cross-talk between G protein-coupled dopamine D5 receptors and ligand-gated $GABA_A$ receptors [31]. It has also been shown that NMDA receptor-mediated functions are modulated by dopamine D1 receptors through direct protein-protein interactions [3]. These studies suggest the possibility that other receptors, including NMDA receptors and $GABA_A$ receptors, may engage in functional cross-talk through a similar mechanism. We suggest that the $GABA_A$ receptor may form a complex with the NMDA receptor that affects NMDA receptor-mediated functions through direct protein-protein interactions. However, fully elucidating the mechanisms underlying the GABA-induced suppression of I_{Asp} would require further study.

The interaction between NMDA and $GABA_A$ receptors may have important implications for central auditory functions. First, this cross-inhibition may exert a neuroprotective effect. When the central auditory system is hyperactive, as is the case during overexposure to sound, GABAergic interneurons may be activated and a large amount of GABA may be released from presynaptic terminals. Increased amount of GABA may then suppress NMDA receptor responses through cross-inhibition, which may prevent the hyperexcitability of postsynaptic neurons. Second, the existence of cross-inhibition between the $GABA_A$ receptor and the NMDA receptor suggests a GABAergic autoregulation mechanism may exist in the central auditory system because studies have shown that activation of $GABA_A$ receptors increases intracellular Ca^{2+} concentrations in some brain regions [32,33]. Third, the inhibition of NMDA receptor-mediated responses by the activation of $GABA_A$ receptors may regulate the temporal properties of excitatory postsynaptic potentials in the IC. Similarly, the inhibition of $GABA_A$ receptor-mediated responses by the activation of NMDA receptors may regulate the temporal properties of inhibitory postsynaptic potentials in the IC. Thus, the interaction between these two types of receptors may play a role

in maintaining the balance between inhibition and excitation in this system. Indeed, the inhibition of synaptically activated NMDA receptors by the activation of $GABA_A$ receptors has been reported to be important for maintaining the temporal precision of responses in the central auditory system [34]. Taken together, the presence of cross-talk between these two types of channels suggests that the mechanisms mediating information processing in the central auditory system may be more complex than previously believed.

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